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An ESAT-6:CFP10 DNA vaccine administered in conjunction with *Mycobacterium bovis* BCG confers protection to cattle challenged with virulent *M. bovis*

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Abstract

The potency of genetic immunization observed in the mouse has demonstrated the utility of DNA vaccines to induce cell-mediated and humoral immune responses. However, it has been relatively difficult to generate comparable responses in non-rodent species. The use of molecular adjuvants may increase the magnitude of these suboptimal responses. In this study, we demonstrate that the co-administration of plasmid-encoded GM-CSF and CD80/CD86 with a novel ESAT-6:CFP10 DNA vaccine against bovine tuberculosis enhances antigen-specific cell-mediated immune responses. ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinated animals exhibited significant ($p < 0.01$) antigen-specific proliferative responses compared to other DNA vaccinates. Increased expression ($p \leq 0.05$) of CD25 on PBMC from ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates was associated with increased proliferation, as compared to control DNA vaccinates. Significant ($p < 0.05$) numbers of ESAT-6:CFP10-specific IFN-γ producing cells were evident from all ESAT-6:CFP10 DNA vaccinated animals compared to control DNA vaccinates. However, the greatest increase in IFN-γ producing cells was from animals vaccinated with ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA. In a low-dose aerosol challenge trial, calves vaccinated as neonates with *Mycobacterium bovis* BCG and ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA exhibited decreased lesion severity in the lung and lung-associated lymph nodes following viruluent *M. bovis* challenge compared to other vaccinated animals or non-vaccinated controls. These data suggest that a combined vaccine regimen of *M. bovis* BCG and a candidate ESAT-6:CFP10 DNA vaccine may offer greater protection against tuberculosis in cattle than vaccination with BCG alone.

Keywords: Adjuvant; Neonate; Tuberculosis

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1. Introduction

DNA vaccination of mice has demonstrated the utility of genetic immunization as a means of inducing robust cell-mediated and humoral responses to encoded antigens [1,2]. Development of successful, practical DNA vaccination in humans and other outbred non-rodent species has been hampered by several factors [3–5]. To induce significant immune responses, vaccinates are often subjected to several immunizations comprised of relatively large amounts of plasmid DNA [6–8]. Individuals vaccinated with DNA are relatively weak responders [6,7]. Molecular adjuvants such as cytokines, costimulatory molecules and immunostimulatory DNA sequences have been used to increase the potency of DNA vaccine-induced immune responses in non-rodent species [9–15].

Following intramuscular (IM) injection of a DNA vaccine, plasmid DNA is internalized by dendritic cells (DCs) and muscle cells. Myocytes express genes encoded on plasmids *in vitro* and *in vivo* [16,17]; however, these cells cannot initiate an immune response via naïve T cells, even when transfected with an appropriate array of costimulatory molecules or cytokines [18]. Effects of DNA vaccination are still apparent following ablation of tissue surrounding the injection site [19]. Plasmid DNA has been isolated directly from DCs within the draining lymph nodes and skin following IM or intradermal inoculation [20]. In addition, DCs can be directly transfected following IM plasmid immunization [21], suggesting that DCs are key mediators of DNA vaccine-induced immune responses. Strategies to manipulate DCs to increase the potency of DNA vaccination, include the use of growth factors such as granulocyte macrophage-colony stimulating factor (GM-CSF) [1,4,22] or *fms*-like tyrosine kinase-3 ligand (Flt-3L) [9,23,24]. In mice and humans, administration of these growth factors, used alone or in combination, increases numbers of DC's [25–29]. Bovine bone marrow cells cultured with GM-CSF alone, GM-CSF + IL-4 or GM-CSF + IL-4 + Flt-3L display morphological characteristics typical of DCs [30]. Experiments in cattle have shown that the co-administration of plasmid-encoded GM-CSF and Flt-3L enhances bovine $CD4^+$ T cell responses to an *Anaplasma marginale* major surface protein-1 (MSP-1) DNA vaccine [9]. The observed enhancement was attributed to an increase in DC recruitment at the site of immunization after growth factor treatment [9].

Early secretory antigenic target-6 kDa (ESAT-6) protein and culture filtrate protein 10 (CFP10) are potent immunogens encoded by region of difference-1 (RD1) in tuberculous mycobacteria [31] and are absent in *Mycobactrium bovis* bacille Calmette-Guerin (BCG) [32,33]. The loss of RD1 is associated with the attenuation of BCG [34,35]. Although no precise function has been attributed to these proteins, their expression correlates with an increased cytolytic ability of *M. tuberculosis* [36]. ESAT-6 is recognized by bovine T cells during the early phase of infection [37], resulting in the release of IFN-γ [37,38], making it an attractive candidate for vaccine development. ESAT-6 and CFP10 exist as a heterodimer in a 1:1 complex *in vivo* [39]. *In vitro*, the use of an ESAT-6:CFP10 fusion protein induces high levels of IFN-γ production [40] and robust proliferative responses by T cells from *M. bovis*-infected cattle.

In our previous study, we assessed the potential of CpG ODN, CD80, CD86 and CD154 to enhance the efficacy of a candidate bovine tuberculosis DNA vaccine directed against ESAT-6 [10]. Co-administration of CpG ODN with either CD80/CD86 or CD154, in a single prime/boost vaccination regimen, enhanced ESAT-6-specific IFN-γ responses as compared to non-vaccinated control animals [10]. However, following aerosol challenge with *M. bovis*, only animals vaccinated with CD80/CD86 demonstrated decreased lesion severity in the lungs and associated lymph nodes [10]. Collectively, this earlier study demonstrated that co-administration of CD80/CD86 is superior to CD154 in augmenting DNA vaccine-induced protection following aerosol challenge with *M. bovis* [10]. In the present study, we evaluated immune responses generated against a candidate ESAT-6:CFP10 DNA vaccine and assessed the ability of plasmid-encoded GM-CSF to further augment the adjuvant activity of plasmid-encoded CD80/CD86 in response to a novel ESAT-6:CFP10 DNA vaccine. In addition, we evaluated the use of a *M. bovis* BCG prime + ESAT-6:CFP10 DNA vaccine prime/boost strategy to induce protection against a low-dose aerosol challenge. Our results indicate that the co-administration of plasmids encoding GM-CSF and CD80/CD86 molecules enhances bovine immune responses to genetic immunization and that this ESAT-6:CFP10 DNA vaccine administered in conjunction with a *M. bovis* BCG prime confers protection against experimental bovine tuberculosis to a greater level than DNA vaccination or BCG vaccination alone.

2. Materials and methods

2.1. Plasmid DNA and recombinant protein production

Plasmids encoding bovine CD80 and bovine GM-CSF were kindly provided by Dr. Chris J. Howard (Institute of Animal Health, Compton, UK). The construction of bovine CD80 and GM-CSF plasmids was described previously [41,42]. For CD86 plasmid construction, total RNA was obtained from bovine PBMC using Trizol Reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed to cDNA using oligo dT 16 (Perkin-Elmer, Branchburg, NJ) according to the manufacturer's instructions. The cDNA was used to amplify an entire open reading frame of bovine CD86 by polymerase chain reaction (PCR) using primers FWD (5′-ACAGCAGAAATAACGAAAATGCG) and REV (5′-CATGGCGTTTACTCTTTAATTACA). PCR conditions were 94 °C (10 min), followed by 35 cycles of 94 °C (15 s), 60 °C (15 s) and 72 °C (2 min). PCR products were cloned into pCR3.1 using T4 ligase (Invitrogen). Sequence and

direction of the insert was confirmed by automated DNA sequencing.

The ESAT-6:CFP10 vaccine plasmid was constructed using the vector VR1020 (Vical Inc., San Diego, CA). The sequence encoding the protein fusion of ESAT-6:CFP10 was amplified from plasmid pISM2202 [40] using primers "ES-CFP-U-Not" (5′-AATGCGGCCGCATATGGGGGGG) and "ES-CFP-L-EcoR1" (5′-GCTGAATTCCGAAGCCCATTTGC). The PCR product was gel purified, digested with *Not*I and *Eco*RI and ligated into, a derivative of plasmid VR1020, plasmid pISM2214. The resulting plasmid was designated plasmid pISM2215 (VR1020:6-His:ESAT-6:CFP10). The DNA sequence and frame orientation was confirmed by DNA sequence analysis. Plasmid DNA for immunization was chemically transformed into competent *Escherichia coli* TOP10 (Invitrogen). Plasmid DNA was purified using the Qiagen Plasmid Giga kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Purified recombinant protein was obtained following induction of TOP10 cells containing pISM2202 by metal chelate chromatography as described [40], dialyzed overnight at 4 °C in PBS and quantified by the Bradford assay.

2.2. Bacteria

M. bovis strain 1315 and attenuated *M. bovis* BCG strain Pasteur were grown in Middlebrook 7H9 media supplemented with 10% oleic acid–albumin–dextrose complex (Difco, Detroit, MI) plus 0.05% Tween 80 (Sigma, St. Louis, MO) as described previously [10]. *M. bovis* 1315 was originally isolated in 1995 from a naturally infected white-tailed deer [43]. Vaccine and challenge inocula consisted of mid-log growth phase mycobacteria. Bacilli were enumerated using serial dilution plate counting on Middlebrook 7H11 selective media (Becton Dickinson, Cockeysville, MD).

2.3. Animals and immunizations

All immunizations and challenge components of the study were conducted at the National Animal Disease Center, Ames, IA. Prior to experimentation, animal-related procedures were approved by the Institutional Animal Care and Use Committee of the National Animal Disease Center.

2.3.1. DNA vaccine trial #1

Twenty-five castrated, 4-month-old Holstein bull calves, were selected for their negative reactivity to purified protein derivative (PPD) from *M. avium* (PPDa) and *M. bovis* (PPDb), as well as rESAT-6:CFP10. Briefly, whole blood was incubated for 24 h *in vitro* in the presence of PPDa (10 μg/ml), PPDb (10 μg/ml) (Biocor Animal Health, Omaha, NE) or rESAT-6:CFP10 (10 μg/ml). Supernatants were harvested and antigen-specific IFN-γ production was determined using the Bovigam ELISA kit (Prionics Ag, Schlieren, Switzerland). Animals negative for previous exposure to mycobacterial antigens were then assigned randomly to the following experimental treatment groups: (1) control DNA (empty plasmid vector pcDNA3.1) ($n=5$); (2) ESAT-6:CFP10 DNA vaccination ($n=5$); (3) ESAT-6:CFP10 + GM-CSF DNA vaccination ($n=5$); (4) ESAT-6:CFP10 + CD80/CD86 DNA vaccination ($n=5$); (5) ESAT-6:CFP10 + GM-CSF + CD80/CD86 ($n=5$). DNA vaccinates were immunized intramuscularly in the right mid-cervical region with 2 mg of total plasmid DNA emulsified in 2 ml of incomplete Freund's adjuvant (IFA). All experimental vaccines contained 1 mg of ESAT-6:CFP10 encoding plasmid. The total amount of plasmid DNA was normalized to 2 mg by additional plasmids depending on the treatment. Cattle received an identical booster dose of vaccine 20 days following primary immunization.

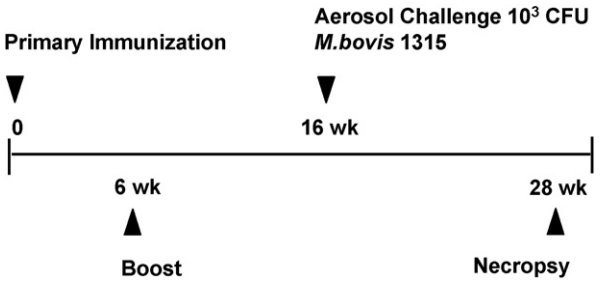

Fig. 1. Timeline detailing events during DNA vaccine trial #2. Calves receiving *Mycobacterium bovis* BCG were immunized with 1×10^6 CFU *M. bovis* BCG strain Pasteur at the time of primary immunization. Animals vaccinated with BCG + DNA or DNA alone received a primary dose of plasmids encoding ESAT-6:CFP10, GM-CSF, CD80 and CD86 in IFA, and an identical booster dose 6 weeks post-immunization. Sixteen weeks after primary immunization, cattle received 10^3 CFU of virulent *M. bovis* 1315 by aerosol and were euthanized 12 weeks after challenge.

2.3.2. DNA vaccine trial #2

Twenty-five Holstein calves less than 6 days of age were randomly assigned to the following experimental treatment groups: (1) non-vaccinated controls ($n=6$); (2) *M. bovis* BCG strain Pasteur vaccination ($n=5$); (3) ESAT-6:CFP10 + GM-CSF + CD80/CD68 DNA vaccination ($n=5$); (4) *M. bovis* BCG strain Pasteur + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccination ($n=5$). At 1 week of age, DNA vaccinates were immunized subcutaneously with 2 mg of total plasmid DNA emulsified in 2 ml of IFA in the left mid-cervical region and received a booster dose at 6 weeks of age (Fig. 1). At the time of the first immunization, animals received 1.0×10^6 CFU *M. bovis* BCG strain Pasteur subcutaneously in the right mid-cervical region.

2.4. Aerosol challenge of vaccinated cattle

Low-dose aerosol challenge of cattle with 1.0×10^3 CFU *M. bovis* 1315 was conducted at the National Animal Disease Center as described previously [44].

2.5. Lymphocyte proliferation assays

PBMC were isolated via density centrifugation from buffy-coat fractions of peripheral blood collected in 2× acid–citrate–dextrose. Peripheral blood mononuclear cells (PBMC) (5×10^5) were cultured in triplicate wells of round-bottom 96-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ) in a total volume of 200 μl. Medium was complete RPMI 1640 (cRPMI) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids (Sigma Chemical Co., St. Louis, MO), 2% essential amino acids (Sigma), 1% sodium pyruvate (Sigma), 50 μM 2-mercaptoethanol (Sigma), and 10% (v/v) fetal bovine sera (FBS). PBMC were stimulated *in vitro* with rESAT-6:CFP10 (5 μg/ml), PPDb (5 μg/ml), pokeweed mitogen (PWM) (1 μg/ml) or cRPMI alone. Cells were cultured for 6 days at 37 °C in 5% CO_2 humidified air with the addition of 0.5–1.0 μCi [^{3}H]-thymidine (specific activity, 6.7 Ci/mM, Amersham Life Science, Arlington Heights, IL) during the last 16–20 h of culture. Well contents were harvested onto glass fiber filters with a 96-well plate harvester (EG & G Wallac, Gaithersburg, MD), and the incorporated radioactivity measured by liquid scintillation counting. Treatments were run in triplicate and presented as mean cpm (±S.E.M.).

2.6. IFN-γ ELISA

Cells were cultured in round-bottom 96-well plates in a volume of 200 μl. PBMC (4×10^5) were cultured *in vitro* in the presence of rESAT-6:CFP10 (5 μg/ml), PPDb (5 μg/ml), pokeweed mitogen (PWM) (1 μg/ml) or cRPMI alone for 48 h at 37 °C in 5% CO_2 humidified air. Following incubation, supernatants were removed and stored at −80 °C until analysis. Supernatants were assayed for IFN-γ production using the Bovigam ELISA kit (Prionics) according to instructions provided by the manufacturer. Concentrations (ng/ml) in test samples were quantified by comparing the absorbance of duplicate test samples with the absorbance of standards within a linear curve fit.

2.7. IFN-γ ELISPOT assay

Antigen-specific IFN-γ production was assayed using an ELISPOT as described previously [10]. Anti-bovine IFN-γ monoclonal antibodies (MAb) CC302 and CC330 were kindly provided by Dr. Chris J. Howard. Briefly, 5×10^5 PBMC were added in 100 μl volumes containing either cRPMI alone, rESAT-6:CFP10 (5 μg/ml), PPDb (5 μg/ml) or PWM (1 μg/ml). Plates were incubated for 36 h at 37 °C in 5% CO_2 humidified air. IFN-γ spot-forming cells were enumerated using a standard dissection microscope or an ELISPOT Reader (CTL). For each animal, the mean number of spots of negative control (i.e. cRPMI alone) wells was subtracted from the number of spots in test wells to determine the mean number of antigen-specific IFN-γ spot-forming cells.

2.8. Detection of antigen-specific antibodies

Round-bottom 96-well plates (Falcon, Becton Dickinson) were coated with rESAT-6:CFP10 (1 μg/ml) in carbonate–bicarbonate coating buffer pH 9.6 overnight at 4 °C. Plates were washed three times with 1× phosphate buffered saline plus 0.05% Tween 20 (PBST) (Sigma) and then blocked for 1 h with milk diluent (Kirkegaard Perry Laboratories, Gaithersburg, MD) at 37 °C. Plates were then washed three times with PBST. Sera were diluted 1:100 in PBS and 100 μl added to wells in duplicate for antigen-coated and control wells (i.e. coating buffer alone) for 1 h incubation at 37 °C. Plates were washed three times with PBST before addition of 100 μl goat anti-bovine IgG (H + L) horseradish peroxidase-conjugated (KPL) secondary antibody diluted 1:10,000 in PBS + 0.1% fish gelatin. For isotype analysis, 100 μl of sheep anti-bovine IgG_1 (Serotec, Oxford, UK) or sheep anti-bovine IgG_2 (Serotec), diluted 1:5000 in PBS + 0.1% fish gelatin, was added as secondary antibody. Plates were incubated for 1 h at 37 °C and washed three times. Wells were developed with the addition of 100 μl TMB substrate (KPL) for 5 min at room temperature. The reaction was stopped by addition of 100 μl of 0.18 M sulfuric acid. A_{450} of individual wells was measured with an automated ELISA plate reader (Molecular Devices, Menlo Park, CA) Changes in optical density (ΔOD) readings were calculated by subtracting the mean OD readings for wells receiving coating buffer alone (two replicates) from the mean OD readings for antigen-coated wells (two replicates) receiving the same serum sample.

2.9. Flow cytometry

PBMC (4×10^5 per well) were cultured *in vitro* in the presence of rESAT-6:CFP10 (5 μg/ml) or cRPMI alone for 6 days at 37 °C in 5% CO_2 humidified air in a round-bottom 96-well plate. Following incubation, cells were subsequently pooled according to treatments. Approximately 4×10^5 pooled cells in 200 μl of culture medium were added to individual wells of round-bottom 96-well plates, centrifuged (2 min, $400 \times g$) and resuspended in 100 μl of primary antibody(s) (1 μg/ml in PBS containing 1% FBS and 0.1% sodium azide). Primary antibodies included anti-CD4 (GC50A1), anti-CD8α (BAQ111A), anti-γδ TCR (GB21A, specific for δ chain) and anti-CD25 (CACT116A) (VMRD, Pullman, WA). After 15 min incubation at room temperature, cells were centrifuged and stained with 100 μl of appropriate secondary antibody [fluorescein isothiocyanate (FITC, 1 μg/well)-conjugated goat anti-mouse IgG_2b, IgM (Southern Biotechnology Associates, Birmingham, AL), phycoerythrin (PE, 1 μg/well)-conjugated goat anti-mouse IgG_1]. Cells were incubated for 15 min at room temperature, centrifuged, washed and resuspended in PBS containing 0.04% sodium azide for acquisition (10,000 live gated events) using a FACScan (Becton Dickinson, San Jose, CA; 488 nm laser, two color) flow cytometer. Data analysis was performed

with commercially available software (FlowJo, Tree Star Inc., San Carlos, CA). Data are presented as the mean (±S.E.M.) percentage of cells expressing a marker.

2.10. Gross and histologic analysis

Lungs, mediastinal and tracheobronchial lymph nodes were subjected to a semi-quantitative gross lesion scoring system adapted from Vordermeier et al. [45]. Lung lobes (left cranial, left caudal, right cranial, right caudal, middle and accessory) were scored as follows: (0) no visible lesions; (1) no external gross lesions, but lesions seen upon slicing; (2) <5 gross lesions of <10 mm in diameter; (3) >5 gross lesions of <10 mm in diameter; (4) >1 distinct gross lesion of >10 mm in diameter; (5) gross coalescing lesions. Lymph node lesion severity was scored as follows: (0) no necrosis or visible lesions; (1) small focus (1–2 mm in diameter); (2) several small foci; (3) extensive necrosis.

Tissues collected for microscopic analysis were fixed by immersion in 10% neutral buffered formalin. For microscopic examination, formalin-fixed tissues were processed by standard paraffin-embedment techniques, cut into 5 μm sections and stained with hematoxylin and eosin. Adjacent sections were cut from samples containing caseonecrotic granulomata suggestive of tuberculosis and stained by the Ziehl–Neelsen method for visualization of acid-fast bacilli. Microscopic tuberculous lesions were staged (I–IV) based on adaptations of criteria described by Rhoades et al. [46] and Wangoo et al. [47]. One microscopic section from each sample collected was evaluated microscopically. The pathologist (M.V. Palmer) was blinded to treatment groups during analysis of tissues for gross and histologic lesion scoring. Data are presented as the mean (±S.E.M.) number of granulomas observed for lung and lung-associated lymph node sections.

2.11. Radiographic lesion morphometry

All lung lobes were radiographed after necropsy to substantiate pathology findings. Radiography was performed using a MinXray machine (Model HF-100, Diagnostic Imaging, Rapid City, SD) with 3M Asymetrix Detail Screens and Ultimate 2000 film (3M Animal Care Products, St. Paul, MN). Developed radiographs were scanned to create digital images. Radiographic lesions were then identified, outlined and measured using Image Pro Plus (Media Cybernetics, Silver Spring, MD) software. Affected area was divided by total lung area then multiplied by 100 to determine percent affected lung. Results for individual animals are presented as the mean percent affected lung for all lung lobes.

2.12. Statistical analysis

Data were analyzed by one-way ANOVA using commercially available software (Statview 5.0, SAS Institute Inc., Cary, NC). Pairwise comparisons between experimental groups were performed using Fisher's protected least significant difference and Student's *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Genetic immunization with plasmid DNA encoding ESAT-6:CFP10 + GM-CSF + CD80/CD86 enhances proliferative recall responses

To determine whether encoded GM-CSF enhanced DNA vaccine responses to ESAT-6:CFP10, the ability of bovine PBMC to proliferate following antigenic restimulation was compared among all experimental treatments. Animals immunized with ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA exhibited significant ($p < 0.01$) responses to rESAT-6:CFP10 stimulation as compared to all other groups at two (Fig. 2A) and 4 weeks post-boost (data not shown). Stimulation with PPDb did not result in significant ($p > 0.05$) proliferative responses following vaccination with plasmid-encoded ESAT-6:CFP10 compared to control vaccinated animals (Fig. 2B). Significant ($p < 0.05$) proliferative responses to rESAT-6:CFP10 were evident only in animals vaccinated with co-administered GM-CSF and CD80/CD86. These data suggest that the co-administration of GM-CSF and CD80/CD86 plasmids in addition to an ESAT-6:CFP10 DNA vaccine results in an antigen-specific recall response.

3.2. The frequency of ESAT-6:CFP10-specific IFN-γ-secreting cells increases following vaccination with ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA

To assess the adjuvant effects of plasmid-encoded GM-CSF, administered alone or administered with CD80/CD86 plasmids, we compared the ability of PBMC from different vaccinates to secrete IFN-γ in response to antigenic stimulation. IFN-γ ELISPOT assays were performed at 2 weeks post-boost to evaluate the frequencies of ESAT-6:CFP10-specific IFN-γ-producing cells (Fig. 3). ESAT-6:CFP10 DNA vaccination resulted in increased ($p < 0.05$) frequencies of IFN-γ-producing PBMC compared to control vaccination, as detected by ESAT-6:CFP10-specific IFN-γ ELISPOT. ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates exhibited greater mean numbers of IFN-γ spot-forming cells (SFC) compared to control vaccinates ($p < 0.0001$), ESAT-6:CFP10 + CD80/CD86 DNA vaccinates ($p < 0.05$) and ESAT-6:CFP10 + GM-CSF DNA vaccinates ($p < 0.05$) (Fig. 3). Similarly, although not statistically significant ($p > 0.05$), ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates produced the highest mean amount of IFN-γ, which was approximately 12-fold higher than control vaccinated cattle (data not shown). As expected, *in vitro* PPDb stimulation did not elicit IFN-γ production by ESAT-6:CFP10 DNA or control vaccinates (data not shown). Differences between ELISPOT responses of ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates

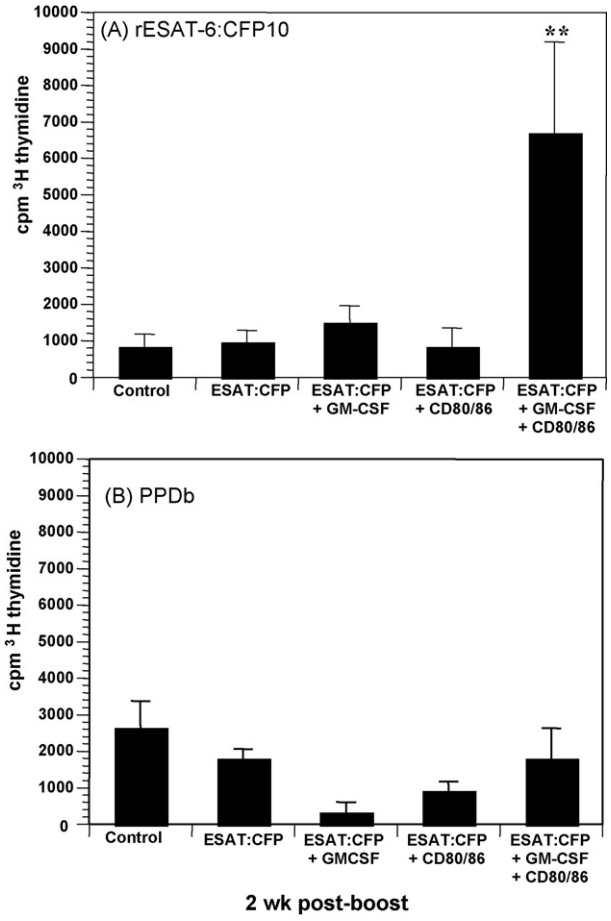

Fig. 2. Proliferative responses from ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates ($n = 5$) are enhanced compared to all other DNA vaccinates following antigenic stimulation at 2 weeks post-boost. PBMC (5×10^5) were cultured *in vitro* for 7 days in the presence of (A) rESAT-6:CFP10 (5 μg/ml), (B) PPDb (5 μg/ml) or media alone. [^{3}H]-thymidine was added to wells the last 18–20 h of culture. **Indicates a significant difference ($p < 0.01$) compared to all other groups. Data represent mean cpm from antigen stimulation cultures minus media alone cpm (±S.E.M.). Similar responses were detected at 4 weeks post-boost. Data were analyzed using ANOVA followed by Fisher's PLSD for post hoc analysis.

and cattle immunized with ESAT-6:CFP10 DNA alone were not significant ($0.05 < p < 0.1$) potentially due to donor variability. Therefore, vaccination with ESAT-6:CFP10 DNA vaccines results in the generation of increased frequencies of antigen-specific IFN-γ-producing cells; however, an additional trend towards greater numbers of SFC following co-administration of plasmid-encoded GM-CSF and CD80/CD86 was also observed.

3.3. CD4$^+$ cells from ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates display increased expression of CD25 following antigenic restimulation

To evaluate whether increased expression of activation molecules was associated with enhanced cell-

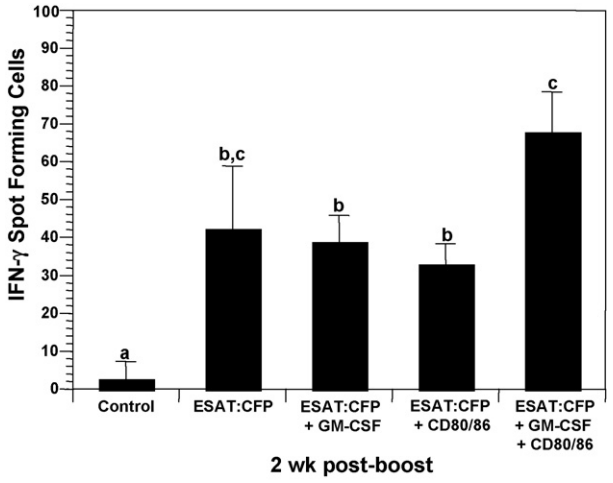

Fig. 3. Genetic immunization with ESAT-6:CFP10 DNA generates increased frequencies of antigen-specific IFN-γ producing cells. Two weeks post-boost, 5×10^5 PBMC were stimulated 36 h in the presence of rESAT-6:CFP10 (5 μg/ml) for the determination of the number of cells producing IFN-γ in response to rESAT:CFP. Data represent the mean (±S.E.M.) number of spot-forming cells from stimulated cultures minus control cultures. PBMC were cultured in triplicate. Letters (a–c) indicate that the treatment means for a specific type of stimulation (i.e. horizontal comparisons) differ ($p < 0.05$). The same letter indicates that responses were not significantly different ($p > 0.05$). Data were analyzed using ANOVA followed by Fisher's PLSD for post hoc analysis.

mediated responses, flow cytometric analysis of CD25 was conducted. Following 6-day culture, CD4$^+$ cells from ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates exhibited increased ($p < 0.05$) expression of CD25 in response to rESAT-6:CFP10 stimulation when com-

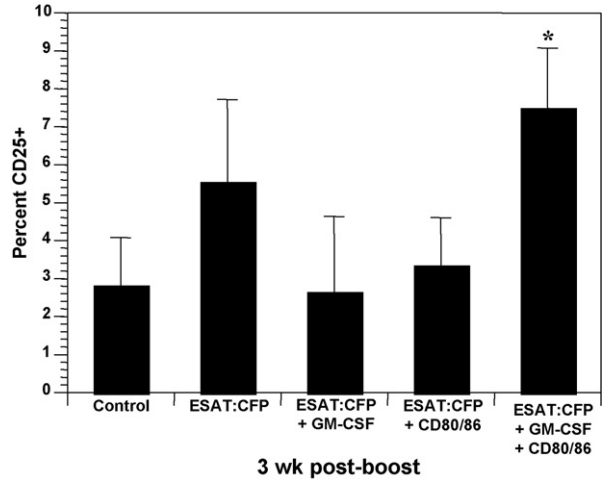

Fig. 4. ESAT-6 + GM-CSF + CD80/CD86 DNA vaccinates ($n = 5$) exhibit increased CD4$^+$ T cell expression of CD25 in response to antigen. Three weeks post-boost, PBMC from vaccinated animals were stimulated for 6 days in the presence of rESAT-6:CFP10 (5 μg/ml) or media alone. Cells were subsequently stained for expression of CD25, indicative of an activated phenotype. Data represent the mean (±S.E.M.) percent expression of CD25 minus control values (i.e. background) from animals in each treatment group.

Table 1
Serum antibody responses to ESAT-6:CFP10 genetic immunization[a]

Vaccination group	ΔOD at A_{450}					
	Total IgG day 0	Total IgG 2 weeks PB	IgG_1 day 0	IgG_1 2 weeks PB	IgG_2 day 0	IgG_2 2 weeks PB
Control ($n=5$)	0.111 (0.035)	0.176 (0.034)	0.016 (0.016)	0.178 (0.072)	0.005 (0.005)	0.138 (0.022)
ESAT-6:CFP10 ($n=20$)	0.140 (0.024)	0.281* (0.029)	0.027 (0.009)	0.370** (0.044)	0.006 (0.002)	0.196 (0.023)
ESAT-6:CFP10 alone ($n=5$)	0.057 (0.021)	0.231 (0.043)	0.000 (0.000)	0.350 (0.094)	0.010 (0.003)	0.214 (0.048)
ESAT-6:CFP10 + GM-CSF ($n=5$)	0.182 (0.066)	0.300** (0.036)	0.052 (0.028)	0.384 (0.089)	0.000 (0.000)	0.233 (0.051)
ESAT-6:CFP10 + CD80/CD86 ($n=5$)	0.151 (0.051)	0.297 (0.093)	0.025 (0.019)	0.402* (0.080)	0.005 (0.003)	0.174 (0.034)
ESAT-6:CFP10 + GM-CSF + CD80/CD86 ($n=5$)	0.170 (0.039)	0.296 (0.061)	0.030 (0.016)	0.347 (0.116)	0.009 (0.004)	0.164 (0.055)

[a] Serum antibody responses to ESAT-6:CFP10 were assessed prior to vaccination (day 0) and at 2 weeks post-boost (PB). Sera were diluted 1:100 and added to ESAT-6:CFP10-coated wells (1 μg/ml) in duplicate. The changes in optical density (ΔOD) readings were calculated by subtracting the mean OD readings for wells receiving coating buffer alone (two replicates) from the mean OD readings for antigen-coated wells (two replicates) receiving the same serum sample. Data are presented as group mean (±S.E.M.). Differs from respective response by controls, $*p<0.1$, $**p\leq 0.05$.

pared to cattle vaccinated with control DNA at 3 weeks post-boost (Fig. 4). Differences in the expression of CD25 on $CD8^+$ and γδ TCR^+ cells were not significant ($p>0.05$) at this timepoint (data not shown). Thus, increased proliferative responses observed from PBMC of ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinated subjects were associated with increased expression of CD25 on CD4-bearing cells.

3.4. Evaluation of antigen-specific humoral responses following ESAT-6:CFP10 DNA vaccination

Antigen-specific serologic responses from animals were evaluated to determine the effects of genetic immunization on antibody responses. Responses (total IgG, IgG_1 and IgG_2) directed toward ESAT-6:CFP were negligible at day 0 of the vaccine trial (Table 1). Analysis of antibody responses 2 weeks post-boost, revealed that increased antibody production was more likely attributable to ESAT-6:CFP10 DNA vaccination rather than the type of molecular adjuvant administered, although a significant ($p\leq 0.05$) increase in total IgG against rESAT-6:CFP10 was seen in animals receiving ESAT-6:CFP10 + GM-CSF-encoding plasmids and a trend ($0.05<p<0.1$) toward increased IgG_1 production observed in cattle vaccinated with ESAT-6:CFP10 + CD80/CD86 DNA. The effect of immunizing with ESAT-6:CFP10 DNA vaccines was more telling when responses from all animals receiving ESAT-6:CFP10 DNA vaccine were pooled. Two weeks following vaccine boost, a trend ($0.05<p<0.1$) toward increased total IgG against rESAT-6:CFP10 was evident in all ESAT-6:CFP10 DNA vaccinates compared to control vaccinates (Table 1). Further analysis of antigen-specific antibody isotypes revealed a significant ($p\leq 0.05$) IgG_1 antibody response against ESAT-6:CFP10 from all DNA vaccinates relative to responses of control animals (Table 1). These data suggest that DNA vaccination with ESAT-6:CFP10 results in the production of antigen-specific humoralresponses.

3.5. M. bovis BCG + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates display reduced lesion severity in the lungs following aerosol challenge with virulent M. bovis

Results from DNA vaccine trial #1 indicated that ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccination elicited more robust immune responses to encoded antigen than all other DNA vaccinates. Therefore, we sought to evaluate whether or not this vaccine combination could confer protection in cattle aerosol challenged with virulent *M. bovis*, and to assess if the addition of BCG enhances plasmid-generated immunity. Previous experiments have determined the location of *M. bovis* after low-dose aerosol challenge of cattle (~120 days) to be primarily focused in the lungs and regional lymph nodes draining the lung. As shown in Table 2, BCG + ESAT-6:CFP10 DNA vaccinates possessed lower gross pathology scores as compared to control animals and other vaccinates. To reinforce these findings, we next employed radiographic lesion morphometry to quantify lung lesions. Non-vaccinated control animals exhibited the highest mean number of radiographic lesions per lung, followed by animals vaccinated with BCG alone and then DNA vaccinates (Table 2). BCG + ESAT-6:CFP10 DNA vaccinates possessed the fewest mean number of lesions compared to all other experimental treatment groups. Similarly, cattle vaccinated with BCG + ESAT-6:CFP10 DNA also displayed the lowest mean percent affected lung following aerosol challenge. Animals vaccinated with ESAT-6:CFP10 possessed comparable levels of percent affected lung and numbers of granulomas upon histologic analysis to BCG + ESAT-6:CFP10 DNA vaccinates, although exhibited a higher mean pathology score and a greater frequency of radiographic lesions in the lung. Numbers of granulomas of the various stages did not vary significantly between treatment groups (data not shown). These results suggest that BCG + ESAT-6:CFP10 DNA vaccination, and to some degree ESAT-6:CFP10 DNA vaccination, achieves reduced pathology of the lung

Table 2
Summary of lung pathology after challenge with virulent *Mycobacterium bovis*

Vaccination group[a]	Gross pathology[b]	Number of radiographic lesions	Animals with radiographic lesions	% Affected lung[c]	Histologic evaluation[d]
Control	1.32 ± 0.70	16.0 ± 9.36	5/5	1.29 ± 1.20	2.4 ± 1.3
BCG	1.44 ± 0.74	8.80 ± 5.15	3/5	0.58 ± 0.30	3.4 ± 2.3
ESAT-6:CFP10	1.20 ± 0.44	8.00 ± 2.72	4/4	0.16 ± 0.03	0.8 ± 0.4
BCG + ESAT-6:CFP10	0.48 ± 0.27	3.60 ± 2.01	3/5	0.12 ± 0.10	0.6 ± 0.4

Data are presented as group mean (±S.E.M.).
[a] Vaccine treatment groups were: non-vaccinated controls ($n = 5$), *M. bovis* BCG strain Pasteur vaccination ($n = 5$), ESAT-6:CFP10 + GM-CSF + CD80/CD68 DNA vaccination ($n = 4$), and *M. bovis* BCG strain Pasteur + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccination ($n = 5$).
[b] Mean disease score: lungs were subjected to a semi-quantitative pathology scoring system described previously [45].
[c] Lung lobes were removed at necropsy and individually radiographed. Lesions were identified on digital images of scanned radiographs, outlined, and measured. Affected area was divided by total lung area then multiplied by 100 to determine percent affected lung.
[d] Mean number of granulomas detected per microscopic section upon histologic evaluation of lung tissue sections.

compared to control animals following virulent *M. bovis* challenge.

3.6. BCG + ESAT-6:CFP10 DNA vaccinates exhibit reduced pathology of the lung-associated lymph nodes

Following aerosol challenge, tracheobronchial and mediastinal lymph nodes were subjected to a previously described pathology scoring system [45]. Results are summarized in Table 3. BCG + ESAT-6:CFP10 DNA vaccinates possessed significantly reduced gross pathology scores of mediastinal and tracheobronchial lymph nodes compared to all other animals (Table 3). Additionally, the mean weights and number of granulomas detected upon histologic evaluation of the lung-associated lymph nodes were the lowest in animals receiving BCG + ESAT-6:CFP10 DNA. Numbers of granulomas of the various stages did not vary significantly between treatment groups (data not shown). These data suggest that vaccination with BCG + ESAT-6:CFP10 DNA results in reduced pathology of the lung-draining lymph nodes following aerosol challenge with virulent *M. bovis*.

4. Discussion

The goal of vaccination is to generate immunologic memory that will provide rapid protection against exposure to a specific pathogen. Following antigenic priming, $CD4^+$ and $CD8^+$ T cells undergo programmed division, resulting in the generation of effector and memory T cell populations [48]. This division occurs independently of further antigenic stimulation [48]. However, factors such as the strength and duration of antigenic stimulation, and level of costimulation affect T cell differentiation and their functional qualities [48]. In this study, we sought to generate improved recall responses to a candidate DNA vaccine (ESAT-6:CFP10) against tuberculosis by addition of plasmid-encoded costimulatory molecules (CD80/CD86) and encoded GM-CSF. In addition, we aimed to evaluate a vaccination regimen in which calves were primed with *M. bovis* BCG and prime/boosted with an ESAT-6:CFP10 DNA vaccine containing plasmid-encoded costimulatory molecules.

Following a single prime/boost regimen, animals immunized with ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccine displayed increased recall responses to antigen

Table 3
Summary of pathology in the lung-associated lymph nodes after challenge with virulent *M. bovis*

Vaccination group[a]	Gross pathology[b]	Lymph node weight (g)	Histologic evaluation[c]
Mediastinal lymph node			
Control	2.70 ± 0.21	29.62 ± 8.68	20.6 ± 7.9
BCG	2.00 ± 0.45	25.12 ± 3.54	23.4 ± 6.1
ESAT-6:CFP10	2.00 ± 0.32	21.42 ± 2.48	24.0 ± 8.6
BCG + ESAT-6:CFP10	0.60 ± 0.40	14.66 ± 1.84	6.8 ± 4.9
Tracheobronchial lymph node			
Control	2.20 ± 0.54	10.36 ± 1.62	24.2 ± 5.9
BCG	1.80 ± 0.58	11.22 ± 2.35	25.2 ± 8.3
ESAT-6:CFP10	2.00 ± 0.58	10.12 ± 1.05	20.2 ± 3.8
BCG + ESAT-6:CFP10	0.60 ± 0.40	7.46 ± 1.22	5.6 ± 3.9

[a] Data are presented as group mean (±S.E.M.), $n = 5$/group. Vaccine treatment groups were: non-vaccinated controls ($n = 5$), *M. bovis* BCG strain Pasteur vaccination ($n = 5$), ESAT-6:CFP10 + GM-CSF + CD80/CD68 DNA vaccination ($n = 4$), and *M. bovis* BCG strain Pasteur + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccination ($n = 5$).
[b] Mean disease score: mediastinal and tracheobronchial lymph nodes were subjected to a semi-quantitative pathology scoring system described previously [45].
[c] Mean number of granulomas detected per microscopic section upon histologic evaluation of lymph node tissue sections.

as compared to control vaccinates. Lymphocytes from ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinated animals proliferated and increased CD25 expression on $CD4^+$ T cells in response to specific antigen. ESAT-6:CFP10 DNA vaccinates possessed increased frequencies of antigen-specific IFN-γ producing cells compared to control vaccinated animals, irrespective of co-administered molecular adjuvants, suggesting that ESAT-6:CFP10 DNA administered in a single prime/boost regimen is capable of inducing immune responses against encoded antigen. However, ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates possessed statistically higher numbers of IFN-γ SFC compared to animals receiving ESAT-6:CFP10 + GM-CSF DNA or ESAT-6:CFP10 + CD80/CD86 DNA vaccines. Additionally, there appears to be an interaction between plasmid-encoded GM-CSF and CD80/CD86, as adjuvant effects on cellular responses were most potent when these molecules were co-administered. Serologic responses from DNA vaccinates revealed modest increases in ESAT-6:CFP10-specific total IgG ($0.05 < p < 0.1$) and IgG_1 ($p \leq 0.05$) production relative to responses of animals vaccinated with control DNA with few observed differences between DNA vaccination groups. These results suggest that co-administration of plasmid-encoded GM-CSF and CD80/CD86 induces enhanced cell-mediated, but not antibody responses to DNA vaccination.

DCs are a specialized cell population linking innate and adaptive immune responses. Adaptive immunity is highly dependent on DC responses, as this population of professional antigen presenting cells is the only subset capable of activating naïve T cells [49]. DCs also spontaneously secrete chemokines that selectively recruit/activate naïve and memory B cells [50]. However, for DCs to acquire these abilities, they must become activated and mature [49]. Several factors affect the maturation of immature DCs. Pathogen-derived signals (LPS, CpG, dsRNA), cytokines (GM-CSF and Flt-3L) and T cell signals (CD154), all induce the activation and maturation of DCs [49]. Activation of DCs results in the acquisition of a mature phenotype, characterized by morphologic changes, high expression of MHC II molecules, CD40 and costimulatory molecules (CD80 and CD86) [49], the latter being critical for providing the second signal needed for naïve T cell activation [51–53]. Therefore, in the present study we explored the ability of plasmid-encoded GM-CSF with or without plasmid-encoded CD80/CD86 to enhance immune responses to genetic immunization in cattle.

The effect of augmenting immune responses by GM-CSF appears to be mediated by providing DCs with a maturation signal so they become efficient antigen presenting cells. *In vitro* addition of GM-CSF to bovine bone marrow cells induces differentiation into DCs [30]. However, without addition of Flt-3L and IL-4, these cells lack potent capacity to stimulate T cells [30]. Further examination revealed that the ability to initiate a T cell response correlated with increased expression of CD80/CD86 and MHC II molecules that was dependent upon the presence of culture with Flt-3L [30]. Indeed, $CD4^+$ T cell responses to DNA vaccination are enhanced in cattle following co-administration of GM-CSF- and Flt-3L-expressing plasmids, explained, in part, by enhanced recruitment of DCs to the injection site [9].

Although direct comparisons cannot be made between our current and previous studies, it is noteworthy that adjuvant effects of CD80/CD86 plasmids were enhanced in the presence of a DC maturation signal (i.e. CpG oligodinucleotides (ODN) or GM-CSF).

In our previous study, we found that the co-administration of CD80/CD86 and CpG ODN to an ESAT-6 DNA vaccine, induced protection following *M. bovis* aerosol challenge [10]. The importance of a DC signal was not evident until the current study, which included controls that did not receive a DC maturation signal (i.e. no GM-CSF). Cell-mediated immune responses from animals vaccinated with ESAT-6:CFP10 + CD80/CD86 DNA were consistently lower than those of ESAT-6:CFP10 + GM-CSF + CD80/CD86 vaccinates. Enhanced immune responses observed in vaccinates, not administered CD80/CD86 and GM-CSF, may be attributable to DC maturation induced by tissue microinjury [2,54], caused by vaccination procedures. Therefore, in our system we speculate that a suboptimal DC maturation signal (GM-CSF alone) is compensated for by the addition of CD80/CD86-encoding plasmids. Based on this hypothesis, DNA vaccine trial #2 evaluated whether or not *M. bovis* BCG could further enhance DNA vaccine-induced protection following aerosol challenge with virulent *M. bovis*.

Following aerosol challenge with virulent *M. bovis*, calves vaccinated with *M. bovis* BCG and ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA exhibited marked reductions in lesion severity in the lung and lung-associated lymph nodes as compared to all other vaccinates. Radiographic analyses confirmed pathology findings in that *M. bovis* BCG + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates possessed the lowest mean percent affected lung and the lowest mean number of radiographic lung lesions compared to non-vaccinated control animals, cattle immunized with a single dose of BCG, or DNA vaccinates. DNA vaccinates possessed a higher level of lung lesion severity and a greater frequency of radiographic lesions, but a comparable level of mean percent affected lung when compared to *M. bovis* BCG + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates. Similarly, upon examination of lung-associated lymph nodes, *M. bovis* BCG + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates possessed the lowest levels of mean lesion severity and a decreased lymph node weight compared to all other vaccinates. These data suggest that the co-administration of *M. bovis* BCG and ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA have additive effects on the reduction of lesion severity following virulent *M. bovis* challenge.

Combinatory approaches have been evaluated using BCG and DNA vaccines to improve vaccination against tuber-

culosis. In cattle, a DNA prime-BCG boost immunization regimen was shown to enhance protection following experimental challenge with *M. bovis* compared to non-vaccinated control animals and animals vaccinated with BCG alone [55]. In addition, Cai et al. recently demonstrated that a DNA vaccine prime followed by a BCG boost resulted in a 10–100-fold reduction of *M. bovis* in the lungs of cattle infected with virulent *M. bovis* compared to animals immunized with DNA alone or BCG alone [56]. In addition, several experiments in mice have been reported to induce enhanced protection using BCG and DNA vaccine strategies. The enhancement of immune responses seen with these combined approaches may be explained by several factors. The addition of DNA vaccines that encode proteins deleted in the attenuation of BCG, may provide important vaccine targets that are present in virulent mycobacteria (i.e. ESAT-6 and CFP10). The inclusion of BCG also presumably provides more T cell epitopes to which immune responses may be mounted. In addition, BCG provides a maturation signal to immature DCs through the involvement of toll-like receptors [57], which may explain our enhancement of DNA vaccine-generated immune responses in the presence of a molecular adjuvant. Further studies will be required to conclusively determine this effect.

To date, *M. bovis* BCG is the only approved vaccine against tuberculosis. However, it has been demonstrated that its efficacy in humans may range from 80% to 0% [58]. The efficacy of BCG vaccination in cattle has been similarly variable (reviewed in Refs. [59,60]). Recent progress regarding BCG vaccination of neonatal cattle and the use of combinatorial vaccines has been promising in finding alternative approaches to standard BCG vaccination. This may be important in controlling the spread of tuberculosis from wildlife reservoirs to cattle and thus, limiting the transmission of virulent mycobacteria to humans, particularly in developing countries or countries with endemic bovine tuberculosis. Natural transmission studies in the field will be needed to accurately determine the efficacy of our vaccine and/or vaccine regimen in preventing the spread of bovine tuberculosis.

In summary, our data demonstrate that ESAT-6:CFP10 DNA vaccination induces potent immune responses, and that co-administration of plasmid-encoded GM-CSF and CD80/CD86 as a single prime/boost regimen enhances immune responses of cattle to ESAT-6:CFP10 genetic immunization, particularly at the level of cell-mediated immunity. The combination of a DC maturation/activation signal and a T cell activation signal appear to be optimal for generating DNA vaccine-induced immune responses in cattle. Finally the combined *M. bovis* BCG and ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccine prime/boost strategy afforded vaccinates the highest degree of protection against *M. bovis*-induced pathology when compared to BCG or a DNA vaccine administered separate of each other by possibly providing an adjuvant signal to potentiate DNA vaccine-induced responses.

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